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CAND1 Promotes PLK4-Mediated Centriole Overduplication and Is Frequently Disrupted in Prostate Cancer[1]

Nina Korzeniewski*, Markus Hohenfellner† and Stefan Duensing*

*Section of Molecular Urooncology, Department of Urology, University of Heidelberg School of Medicine, Heidelberg, Germany; †Department of Urology, University of Heidelberg School of Medicine, Heidelberg, Germany

Abstract

Centrosomes play a crucial role in the maintenance of genome stability by orchestrating bipolar mitotic spindle formation. The centrosome normally duplicates precisely once before mitosis in a process that is extensively regulated by protein degradation including SKP1–Cullin 1 (CUL1)–F-box (SCF) E3 ubiquitin ligase activity. The core SCF component CUL1 has recently been found to be required to suppress the formation of supernumerary centrosomes and centrioles, the core-forming units of centrosomes. Here, we identify the CUL1-interacting protein cullin-associated and neddylation-dissociated 1 (CAND1) as a novel centrosomal protein with a role in centriole duplication control. CAND1 was found to synergize with Polo-like kinase 4 (PLK4), a master regulator of centriole biogenesis, in the induction of centriole overduplication. We provide evidence that CAND1 functions in this process by increasing PLK4 protein stability. Furthermore, mutants of CUL1 that lack the ability to interact with CAND1 and are unable to assemble functional E3 ubiquitin ligase complexes were impaired in their ability to restrain aberrant daughter centriole synthesis. To corroborate a role of CAND1 in human carcinogenesis, we analyzed a series of prostate adenocarcinomas and found altered expression of CAND1 on the mRNA or protein level in 52.9% and 40.8%, respectively, of the tumor samples analyzed. These results highlight the role of altered SCF components in cancer in general and encourage further studies to explore the SCF-CAND1 axis for the development of novel predictive biomarkers and therapeutic approaches in prostate cancer.

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Introduction

Aneuploidy is a frequent finding in human cancer and commonly associated with aggressive tumor phenotypes and malignant progression. Several mechanisms can cause aneuploidy, and in particular, centrosome-mediated cell division errors are believed to play an important role [1,2]. Centrosomes are the major microtubule-organizing centers of most mammalian cells, both during interphase and mitosis [3]. They consist of a pair of centrioles, short barrel-shaped microtubule cylinders, embedded in pericentriolar material [4]. The single centrosome of a nondividing cell duplicates precisely once before mitosis to produce two centrosomes that function as mitotic spindle poles to determine spindle bipolarity and orientation. Cancer cells frequently show aberrant centrosome numbers leading to mitotic spindle abnormalities and chromosome missegregation [5,6]. Precise control of centrosome duplication is hence pivotal for the maintenance of genome integrity during mitosis.

Centrosome duplication is initiated by disengagement of the two preexisting (maternal) centrioles by the coordinated action of Polo-like kinase 1 (PLK1) and separase [7]. Daughter centriole formation is then triggered by the recruitment of PLK4 to the wall of maternal centrioles through interaction with another centriolar protein, CEP152 [8–10]. It has previously been shown that tight regulation of PLK4 by SKP1–Cullin 1 (CUL1)–F-box (SCF) E3 ubiquitin ligase activity and proteasomal degradation is essential to prevent the synthesis of extra daughter centrioles [11]. CUL1 has recently been reported to

Address all correspondence to: Stefan Duensing, MD, Section of Molecular Urooncology, Department of Urology, University of Heidelberg School of Medicine, Medical Faculty Heidelberg, Im Neuenheimer Feld 517, 69120 Heidelberg, Germany.
E-mail: stefan.duensing@med.uni-heidelberg.de
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suppress centriole multiplication, a process where a single maternal centriole assembles multiple daughters concurrently [12].

CUL1 is one of seven human cullins that assemble into cullin–RING E3 ubiquitin ligases (CRLs). It functions as a scaffold protein and interacts with SKP1 at its amino-terminus to recruit a substrate-binding protein (F-box–containing protein) and with RBX1, a RING-domain protein, at its carboxy-terminus to facilitate interaction with an E2 ubiquitin–conjugating enzyme. The various F-box proteins that have been identified to date are responsible for substrate recognition and binding by the complex and hence provide specificity to the poly-ubiquitination process and ultimately targeted protein degradation by the proteasome [13].

Besides this modular organization of most CRLs, a number of regulatory factors have been identified. All cullins can be post-translationally modified by the covalent attachment of the small ubiquitin-like protein NEDD8. Neddylation is associated with enhanced CRL activity, whereas deneddylation, which is mediated by the COP9 signalosome, decreases CRL activity. In addition, the cullin-interacting protein cullin-associated and neddylation-dissociated 1 (CAND1) has been identified to play a role in modulating CRL activity [14,15].

CAND1 is a 120-kDa HEAT repeat–containing protein that inhibits the assembly of multi-subunit E3 ubiquitin ligase complexes. CAND1 only binds unneddylated and, hence, inactive CUL1 and competes with SKP1 for binding to CUL1, thereby generally suppressing SCF ubiquitin ligase activity [14–16]. However, CAND1 has also been reported to be required for CRL function in certain model organisms *in vivo* [17,18]. These contradictory findings have been, at least in part, reconciled by two results. First, it was shown that expression of CAND1 can prevent substrate adapter autoubiquitination [15,19]. Second, CAND1 binding and dissociation was found to promote the assembly and disassembly of SCF complexes, thereby stimulating the activity of certain SCF complexes, as opposed to a global inhibition [20,21].

Here, we show that CAND1 is a centrosomal protein that controls the stability of PLK4, a master regulator of centriole biogenesis. CAND1 was found to synergize with PLK4 to stimulate centriole overduplication and we provide evidence that CAND1 expression is disrupted on both the mRNA and protein levels in a substantial fraction of prostate cancers. These findings underscore the important role of regulators of CRL activity in human cancer in general and encourage further studies for the development of novel biomarkers and therapeutic approaches in prostate cancer.

Materials and Methods

Cell Culture and Transfections

U-2 OS/centrin-green fluorescent protein (GFP) cells (centrin-GFP construct kindly provided by Michel Bornens, Institut Curie, Paris, France) [22] were cultured and transiently transfected as previously described [12]. Plasmids used for cell transfections were myc-tagged CAND1, hemagglutinin (HA)-tagged wild-type CUL1 or HA-tagged mutant CUL1-ΔN53 or CUL1-ΔC22 (kindly provided by Yue Xiong, University of North Carolina, Chapel Hill, NC), or myc-tagged PLK4 (kindly provided by Erich A. Nigg, Biocenter, University of Basel, Basel, Switzerland). Cells were plated onto 60-mm tissue culture dishes and transfected with 2 μg of plasmid DNA using lipofection (FuGENE 6, Roche, Basel, Switzerland) unless indicated otherwise.

Immunologic Methods

Cells were grown on coverslips and processed for immunofluorescence analysis as previously described [23]. For pre-extraction, slides were immersed in 1% Triton-X for 12 minutes before paraformaldehyde fixation. Antibodies used were directed against CAND1 (H-85, rabbit polyclonal directed against amino acid residues 396–480, Santa Cruz, Santa Cruz, CA; or 5D7, mouse monoclonal directed against amino acid residues 1–100; Abnova, Taipei, Taiwan), CEP170 (Invitrogen, Camarillo, CA), or CUL1 (Santa Cruz; all at a 1:50 dilution). Rhodamine Red–conjugated secondary antibody or Cyan Fluorescent Protein–conjugated secondary antibody was used (Jackson Immunoresearch, West Grove, PA).

Immunohistochemical stainings of benign or malignant prostate specimens for CAND1 or CUL1 were performed using a commercially available tissue microarray (US Biomax, Rockville, MD). Briefly, sections were deparaffinized in xylene, rehydrated in a graded ethanol series, and boiled in a microwave oven for 30 minutes in a citrate buffer (pH 6.0) followed by blocking and incubation with primary antibodies against CAND1 (5D7, Abnova, at a 1:50 dilution) or CUL1 (Ab-1, Lab Vision, Kalamazoo, MI, at a 1:200 dilution). Immunoperoxidase detection of primary antibodies was performed using the Histostain-Plus Kit (Invitrogen) according to the manufacturer's recommendations.

For immunoblot analysis, whole protein extracts were obtained as previously described [23]. Immunoblot analysis was performed using antibodies against HA-tag (Covance, Princeton, NJ), myc-tag (Cell Signaling, Dancers, MA), actin (Sigma-Aldrich, St Louis, MO), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz). To assess protein stability, we treated cells with 60 μg/ml cycloheximide (CHX) for the indicated time intervals.

Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reaction (qPCR) analysis of nine normal and 31 cancerous prostate specimens for CAND1 mRNA expression was performed using the TissueScan Prostate Cancer cDNA Array III (Origene, Rockville, MD). Total RNA obtained from pathologist-verified tissues was normalized and validated with β-actin in two sequential qPCR analyses by the manufacturer. cDNAs were resuspended according to the manufacturer's protocol. qPCR was performed using specific primers to *CAND1* (forward: 5′-GCTGATATGTTGAGCAGGCAA-3′, reverse: 5′-ACTGGGGAAGTAGACAGGTCA-3′; IDT, Leuven, Belgium) and measured using the SsoFast EvaGreen Kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Cycling conditions were 95°C (30 seconds, activation), 95°C (5 seconds, denaturation), and 60°C (10 seconds, annealing/extension) for 40 cycles on a Bio-Rad CFX96 Real-Time System run on a C1000 Thermal Cycler (Bio-Rad). Threshold for Cq determination was set according to the manufacturer's protocol. Relative fluorescence units are given.

Flow Cytometry

Cells were analyzed for cell cycle distribution after propidium iodide staining using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

Statistical Analysis

Student's two-tailed *t* test for independent samples and Fisher's Exact Probability test (two-tailed) were used wherever applicable. All centriole quantification experiments were performed at least as three independent experiments with at least 100 cells counted per experiment.

Results

CAND1 Colocalizes with CUL1 at the Centrosome

On the basis of the role of CAND1 as CUL1-interacting protein and the finding that CUL1 is a centrosomal protein, we asked first whether CAND1 may localize to the centrosome. A predominantly centrosomal localization of CAND1 together with a weak nuclear staining was detected in pre-extracted U-2 OS cells stably expressing centrin-GFP (Figure 1*A*). Without pre-extraction, CAND1 showed a nucleocytoplasmic localization in line with previous findings [24]. Using a second antibody directed against a different epitope, we confirmed the centrosomal localization of CAND1 and found CAND1 to colocalize with CUL1 at the centrosome by coimmunofluorescence microscopy (Figure 1*B*). Similar to CUL1 [12], CAND1 was found to localize predominantly to mature maternal centrioles as evidenced by colocalization of CAND1 with CEP170 (Figure 1*C*) and ninein (not shown) [25,26]. The centrosomal localization of CAND1 was dependent on the presence of CUL1 because small interfering RNA (siRNA)-mediated knockdown of CUL1 abolished CAND1 staining (Figure 1*D*). This suggests that CUL1 may be the predominant centrosomal binding partner of CAND1. CAND1 was found to localize to centrioles throughout the cell division cycle (Figure 2). In addition, we found that the second mature centriole, which is present in the G2 phase of the cell division cycle, also acquires CAND1 staining (Figure 2).

On the basis of the idea that CAND1 has, in general, inhibitory activities on the assembly of active SCF E3 ubiquitin ligase complexes, we asked whether ectopic expression of CAND1 can induce centriole multiplication, which has been reported previously in cells in which CUL1 was inhibited, for example, by siRNA or a dominant-negative (DN) CUL1 mutant [12]. We found that transient overexpression of CAND1 led to only a modest increase of U-2 OS/centrin-GFP cells with centriole overduplication (defined as more than four centrioles

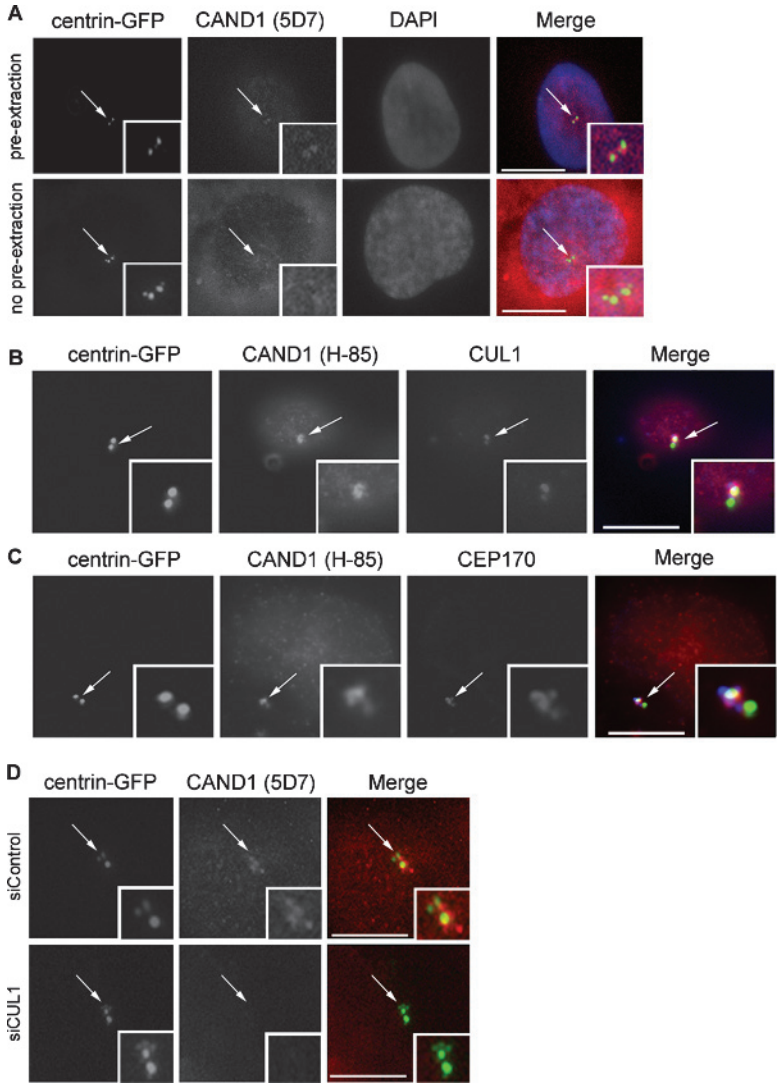


Figure 1. CAND1 is a centrosomal protein and colocalizes with CUL1. (A) Immunofluorescence microscopic analysis of CAND1 using a monoclonal antibody against CAND1 (5D7) in U-2 OS/centrin-GFP cells with pre-extraction (top panels) or without pre-extraction (bottom panels). (B, C) Coimmunofluorescence microscopic analysis of CAND1 and CUL1 (B) and CAND1 and CEP170 (C) in U-2 OS/centrin-GFP cells. A second, polyclonal antibody (H-85) is used. (D) Immunofluorescence analysis of CAND1 (antibody 5D7) in U-2 OS/centrin-GFP cells transfected with either control siRNA or siRNA targeting CUL1. Note the loss of CAND1 expression at centrioles in CUL1-depleted cells. All arrows point at the centrioles shown in insets. All scale bars, 10 μm.

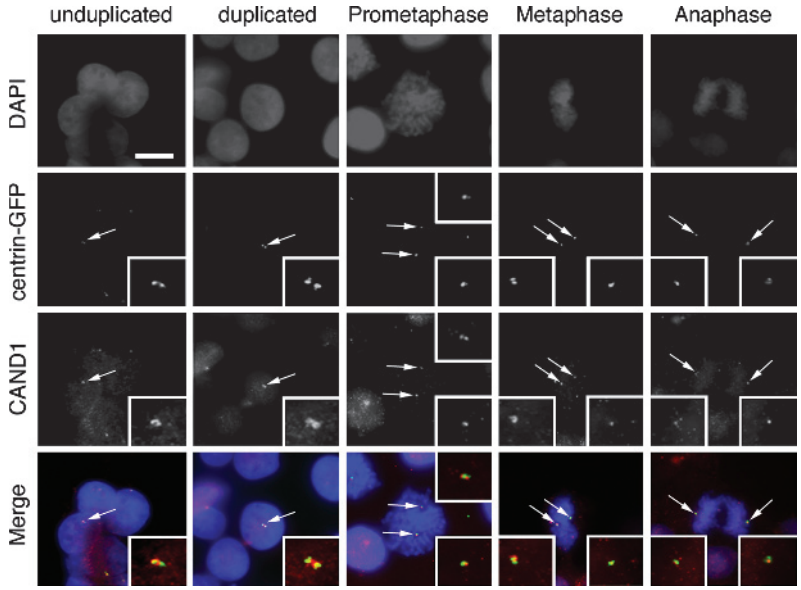


Figure 2. CAND1 expression during the cell division cycle. Immunofluorescence microscopic analysis of CAND1 expression at different cell cycle stages in asynchronously growing U-2 OS/centrin-GFP cells. All arrows point at the centrioles shown in insets. Scale bar, 10 μm.

per cell and at least one maternal centriole with more than one daughter) from 0.5% in controls to 1.7% after 48 hours ($P < .05$, Student's t test for independent samples, two-tailed).

CAND1 forms a clamp around CUL1 to occupy the SKP1 binding site at the N-terminus, whereas the CAND1 HEAT repeats interact with the C-terminus, thereby blocking the CUL1 neddylation site [16]. We therefore tested the effects of two CAND1 binding–deficient CUL1 mutants, in which 53 residues from the amino-terminus (ΔN53) or 22 residues from the carboxy-terminus (ΔC22) [27], respectively, have been deleted, for effects on centriole overduplication (Figure 3, *A* and *B*). Both mutants showed an impaired ability to restrain aberrant daughter centriole biogenesis at maternal centrioles when compared to full-length CUL1 (Figure 3*B*). Transient overexpression of CUL1-ΔC22 led to an increase of the proportion of cells with genuine centriole overduplication from 0.25% in controls to 3.4% ($P < .05$), whereas ectopic expression of CUL1-ΔN53 caused an increase that did not reach statistical significance (3%; $P > .05$). The lack of significant centriole overduplication in CAND1-expressing cells and the moderate increase in cells overexpressing the two CUL1 deletion mutants were not related to the fact that cells were in a nonpermissive state because pretreatment with aphidicolin to induce an S phase arrest could not increase centriole overduplication when compared to empty vector controls (data not shown).

To address the question of how the CUL1 mutants increase centriole overduplication, we first performed a cell cycle analysis, but no significant changes in the cell cycle distribution were detected (Figure 3*C*).

Because PLK4 has been identified as a key CUL1 substrate in centriole duplication control, we analyzed PLK4 protein expression after transient overexpression of CUL1 mutants followed by a 6-hour CHX block to determine protein stability. We found that transient overexpression of mutant CUL1-ΔN53 did not lead to increased PLK4 protein expression after 48 hours but that the mutant CUL1-ΔN53 stabilized PLK4 level after 6-hour CHX (Figure 3*D*). In contrast, overexpression of CUL1-ΔC22 led to an increase of PLK4 protein expression after 48 hours, but there was no significant stabilization detectable after a 6-hour CHX block. Together, these findings suggest that CAND1 binding–deficient CUL1 mutants can induce a modest increase of centriole overduplication that, at least for the C-terminal mutant CUL1-ΔC22, involves up-regulation of the PLK4 protein.

CAND1 Synergizes with PLK4 to Induce Centriole Overduplication through Enhanced PLK4 Protein Stability

Because the previous results suggest a role of PLK4 protein expression in the relaxation of centriole duplication control, we next asked whether CAND1 may synergize with PLK4 to stimulate centriole overduplication. We tested whether CAND1 may modulate PLK4 function when both are coexpressed. For this experiment, we used the ability of PLK4 to induce full centriole multiplication, i.e., "centriole flowers" (Figure 3*F*) [28,29], as experimental readout. When we transiently overexpressed CAND1 in U-2 OS/centrin-GFP cells for 24 hours and then transfected cells with increasing amounts of PLK4 plasmid DNA for an additional 24 hours, we found that cells coexpressing CAND1 and PLK4 showed significantly higher levels of centriole multiplication than cells expressing PLK4 alone (10.7% *vs* 4.5% with 0.1 μg of PLK4 DNA transfected, $P < .005$ and 28.2% *vs* 13.1% with 0.5 μg of PLK4 DNA transfected, $P < .001$; Figure 3*G*).

To further corroborate these results, we determined the protein stability of PLK4 in the presence or absence of CAND1. Using a 6-hour CHX block experiment, we found that CAND1 overexpression led to an increased protein stability of ectopically expressed PLK4 (Figure 3*H*). Taken together, these results show that although CAND1 is unable to stimulate a significant increase of cells with centriole overduplication alone, it can significantly promote PLK4-induced centriole overduplication.

CAND1 mRNA and Protein Expression Is Frequently Disrupted in Prostate Cancer

In general, little is known about the role of CAND1 in human tumorigenesis [24]. However, CAND1 has previously been shown to be subject to microRNA (miRNA)-mediated regulation in a prostate cancer cell line [30]. We therefore asked whether CAND1 mRNA expression levels may be altered in a series of primary prostate adenocarcinomas.

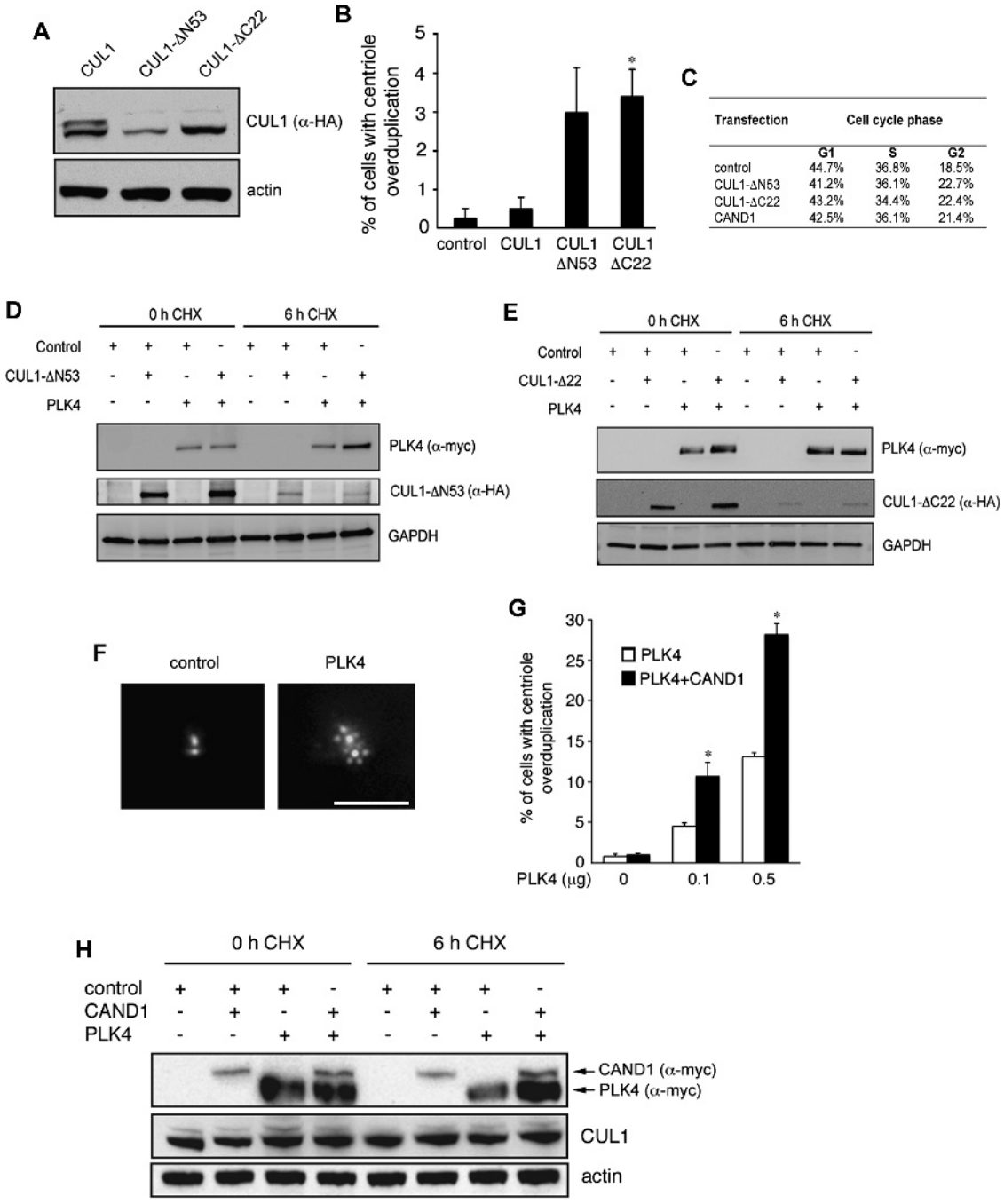


Figure 3. CAND1 stabilizes PLK4 and synergizes with PLK4 to induce centriole overduplication. (A) Immunoblot analysis of U-2 OS/centrin-GFP cells for CUL1 and CUL1 deletion mutants. Immunoblot for actin is shown for protein loading. (B) Quantification of the percentage of U-2 OS/centrin-GFP cells with centriole overduplication (more than four per cell and at least one maternal centriole with more than one 1 daughter) following transient transfection with empty vector (control), CUL1, or CUL1 mutants. Mean and standard errors of three independent experiments are shown. Asterisks indicate statistically significant differences ($P < .05$, Student's t test for independent samples, two-tailed). (C) Cell cycle distribution after transient transfection of U-2 OS/centrin-GFP cells as assessed by flow cytometry. (D, E) Immunoblot analysis of U-2 OS/centrin-GFP cells for PLK4 (anti-myc) or CUL1 mutants (anti-HA) 48 hours after transfection with PLK4-myc, CUL1-ΔN53-HA or CUL1-CΔ22-HA transfection (0-hour CHX), or after 6-hour CHX block. GAPDH is shown to demonstrate protein loading. (F) Fluorescence microscopic analysis of U-2 OS/centrin-GFP cells following transfection with either empty vector (control) or PLK4. Note the concurrent formation of multiple daughter centrioles at single maternal centrioles ("centriole flower"), which is highly characteristic for PLK4 overexpression (right panel). Scale bar, 5 μm. (G) Quantification of the percentage of U-2 OS/centrin-GFP cells with centriole overduplication after transfection with PLK4 alone (open bars, μg of PLK4 plasmid DNA transfected as indicated) or in combination with CAND1 (black bars). Mean and standard errors of three independent experiments are shown. Asterisks indicate statistically significant differences ($P < .005$, Student's t test for independent samples, two-tailed). (H) Immunoblot analysis of U-2 OS/centrin-GFP cells for CAND1 and PLK4 (anti–myc tag), CUL1 or actin after transient transfection of cells with empty myc tag vector (control), CAND1-myc, and/or PLK4-myc and treatment of cells with CHX (60 μg/ml) for the indicated time intervals. Note the increased protein expression of PLK4 in cells cotransfected with CAND1 after 6-hour CHX in comparison to the decreased protein expression when PLK4 is expressed individually.

We performed a quantitative reverse transcription–PCR analysis of nine normal and 31 cancerous prostate samples using a commercially available qPCR gene expression array containing a series of normalized and validated cDNA samples (Figure 4). The mean Cq for normal prostate samples was 35.69 ± 1.23 (SD). Of the 31 prostate cancer samples, one tumor (3.2%) showed a Cq value greater than mean + 3 SD (i.e., 39.38 cycles) indicating reduced mRNA expression in comparison to controls, whereas 15 tumors (48.4%) showed a Cq value below mean – 3 SD (i.e., 35.69 cycles) indicating increased mRNA expression (Figure 4). These findings indicate that CAND1 is transcriptionally deregulated, predominantly upregulated, in a fraction of prostate cancers.

To further corroborate and extend these results, we performed an immunohistochemical analysis of normal (n = 10), hyperplastic (n = 19), and malignant (n = 49) prostate specimens for CAND1 and CUL1 using a commercially available tissue microarray (Figure 5). Most normal and hyperplastic specimens show a weak-to-moderate nucleocytoplasmic CAND1 staining (90% and 94.7%, respectively; Figure 5, *A* and *B*), whereas adenocarcinomas showed a loss of CAND1 expression in 14.3% of tumors and overexpression in 26.5% of tumors. CUL1 immunohistochemistry of an adjacent section of the tissue microarray showed likewise a predominantly weak-to-moderate staining in normal and hyperplastic specimens (80% and 78.9%, respectively; Figure 5, *A* and *B*), whereas adenocarcinomas showed loss of expression in 4.1% and an overexpression in 36.7% of tumors. The correlation between an altered (loss or overexpression) CAND1 expression and malignancy was statistically significant ($P < .005$), whereas the correlation between altered CUL1 expression and malignancy did not reach statistical significance (Figure 5*B*).

Discussion

The results presented here highlight a novel and important role of the CRL regulatory protein CAND1 in centrosome duplication control and cancer. Our findings provide evidence for a frequent disruption of CAND1 expression on both the mRNA and protein level in prostate cancer. When overexpressed *in vitro*, CAND1 had only little effect on centriole biogenesis. However, we detected a pronounced synergistic effect with PLK4, a master regulator of centriole biogenesis [29], on aberrant centriole biogenesis. Mechanistically, we found that PLK4 protein was significantly stabilized when CAND1 was overexpressed. These results suggest that overexpression of CAND1 may promote malignant progression by stabilizing PLK4, thereby enhancing centrosome overduplication and mitotic defects.

To corroborate the last point, we analyzed the 13 prostate cancer samples with CAND1 overexpression and found that seven also showed upregulated PLK4 expression (data not shown). We believe that these data need to be confirmed in a more stringent fashion and, importantly, on a cell-by-cell basis, but these preliminary data point to an association between CAND1 expression and PLK4 protein expression in cancer. Whether up-regulation of CAND1 leads to PLK4 accumulation simply through inhibition of its SCF-mediated ubiquitination or whether disruption of proper assembly and disassembly cycles of CRLs as previously suggested [20,21] plays a role remains to be determined (see also below).

A number of questions remain to be answered including why CAND1 overexpression alone does not lead to centriole overduplication and how the C-terminal and N-terminal CUL1 mutants subvert centriole duplication control.

CAND1 was only found to stabilize ectopically expressed PLK4 after a CHX block similar to mutant CUL1-ΔN53, which, in contrast to CAND1, did increase centriole overduplication to a certain degree. Mutant CUL1-ΔC22 was found to cause an increase of PLK4 already at 48-hour post-transfection together with centriole overduplication.

There are a number of points that need to be taken into consideration when interpreting these perplexing results. First, the overall level of centriole overduplication is lower in CAND1- or CUL1 mutant–expressing cells than what has previously been detected in cells transfected with DN CUL1 mutant [12]. This makes it unlikely that CAND1 simply functions as a CUL1 inhibitor that would lead to a more pronounced increase of centriole overduplication. However, aberrant PLK4 protein levels after ectopic expression could stimulate increased E3 ubiquitin ligase activity to counterbalance the overabundance of PLK4. Under such conditions, increased CAND1 levels could impede SCF ubiquitin ligase assembly, thereby stabilizing PLK4 and promoting centriole overduplication as shown here. The presence of such feedback loops to control steady-state protein expression has recently become evident [31]. CAND1 can also have positive

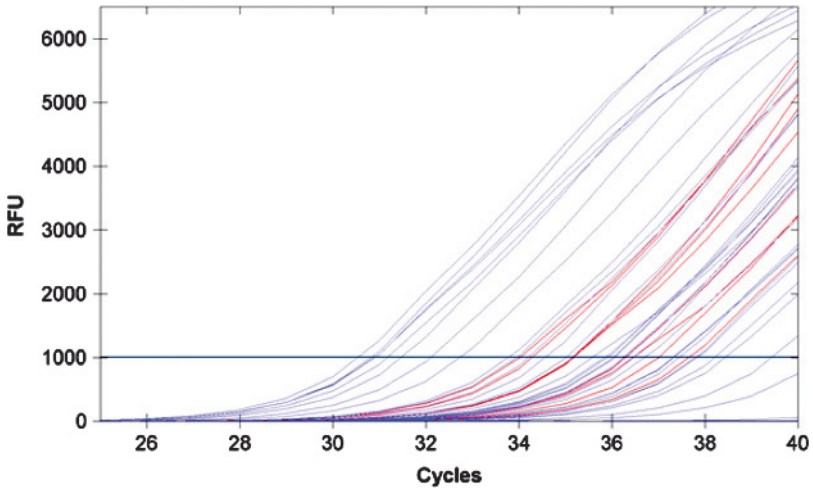
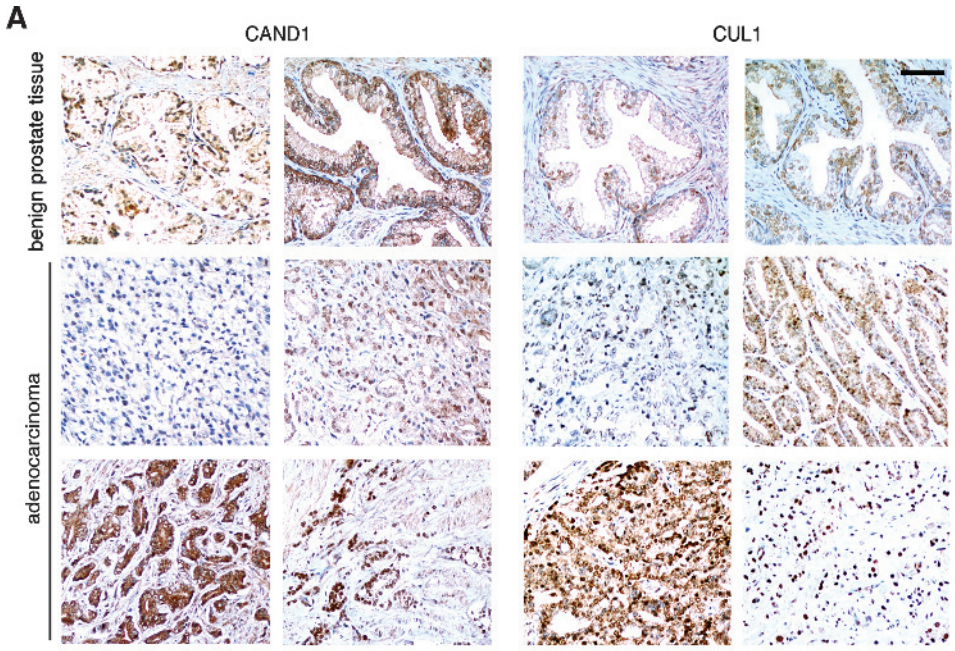


Figure 4. Disruption of CAND1 mRNA expression in prostate cancer. qPCR analysis of nine normal and 31 cancerous prostate specimens for CAND1 mRNA expression. Relative fluorescence units are given. Red amplification curves represent normal control tissue; blue amplification curves represent prostate cancer samples.



B

Diagnosis	Staining	CAND1	CUL1
Normal (n=10)	overexpression	0	2 (20%)
	weak-moderate	9 (90%)	8 (80%)
	loss	1 (10%)	0
Hyperplasia (n=19)	overexpression	1 (5.3%)	1 (5.3%)
	weak-moderate	18 (94.7%)	15 (78.9%)
	loss	0	3 (15.8%)
Adenocarcinoma (n=49)	overexpression	13 (26.5%)	18 (36.7%)
	weak-moderate	29 (59.2%)	29 (59.2%)
	loss	7 (14.3%)	2 (4.1%)
		$p \leq 0.005$*	$p > 0.05$

*Association between altered expression levels and malignancy (Fisher's Exact Probability Test, two-tailed)

Figure 5. CAND1 protein is overexpressed in a subset of prostate cancers. (A) Immunohistochemical analysis of benign prostate tissue specimens and prostate adenocarcinomas for CAND1 or CUL1. Note the predominantly weak-to-moderate expression in the benign tissue samples. Note the loss (top left panel of adenocarcinomas), weak-to-moderate staining (top right), or overexpression (bottom panels) of CAND1 and CUL1, respectively, in prostate adenocarcinoma samples. Scale bar, 50 μm. (B) Overview of immunohistochemical staining results.

effects on E3 ubiquitin ligase assembly, but we believe that our results in the overexpression scenario speak against this possibility, which does not preclude this possibility under conditions where PLK4 is not ectopically expressed.

With respect to the two CUL1 mutants, several additional aspects also have to be considered. Both CUL1 deletion mutants fail to interact with CAND1 [27]. This could, in theory and under the assumption that CAND1 functions primarily as a CUL1 inhibitor, lead to a situation in which SCF complexes become "hyperactive" because of disinhibition. This should lead to a reduced PLK4 expression and no centriole overduplication or even suppression of this process. Nevertheless, we detected a moderate but reproducible and, in the case of CUL1-ΔC22, even statistically significant induction of centriole overduplication. As mentioned above, binding to CAND1 can also positively influence E3 ubiquitin assembly and lack of binding could, hence, impair protein degradation, which, at least for PLK4 levels immediately 48 hours after transfection, is what our results show for the C-terminal CUL1 mutant. Nonetheless, the N-terminal mutant does stimulate centriole overduplication, albeit not in a significant manner, but no increase of PLK4 at 48 hours after transfection level is detectable.

To explain these apparent discrepancies, we need to discuss the fact that these CUL1 mutants are defective in other ways as well. The C-terminal sequence of CUL1 has been reported to be required for its nuclear localization, and CUL1-ΔC22 was found to have a severely reduced nuclear localization and to accumulate in the cytoplasm [27]. In addition, nuclear localization was found to promote CUL1 neddylation and full activation of its ubiquitin ligase activity [27]. The altered subcellular abundance together with impaired ubiquitin ligase activity of CUL1-ΔC22 could readily impact on the degradation of PLK4. PLK4 is predominantly centrosomal, i.e., outside the nucleus, and rapidly accumulates at the centrosome when its degradation is abrogated [12]. Hence, cytoplasmic mislocalization of CUL1-ΔC22 could rapidly lead to increased PLK4 levels at 48 hours and before the CHX block.

The N-terminal CUL1 deletion mutant can be expected to localize to the nucleus and to become neddylated, yet it fails to bind SKP1 [14], which associates with the N-terminus of CUL1. This could impact the turnover of PLK4 protein after CHX block as shown in Figure 3*D* similar to CAND1.

Why the N-terminal CUL1 mutant can still, to a certain degree, albeit not in a statistically significant manner, induce centriole overduplication without an apparent PLK4 protein increase at 48 hours after transfection is unclear. One possibility is that this mutant affects the protein expression of cofactors for PLK4-induced centriole overduplication that are also targets of CUL1-mediated degradation without apparent change of PLK4 total level, for example, cyclin E [12]. Future experiments will address this interesting possibility.

Regarding the mechanism of CAND1 up-regulation in prostate cancer, we provide evidence that at least in some tumors altered gene transcription may play a role. A previous study has shown that that up-regulation of microRNA miR-148a can lead to a reduced CAND1 expression in a prostate cancer cell line [30]. Because some of the tumors analyzed here show a loss of CAND1 mRNA and protein expression, microRNA-mediated modulation of CAND1 expression is a possibility that warrants further testing. Nevertheless, transcriptional upregulation appears to be an important mechanism of CAND1 deregulation in primary prostate cancers.

Taken together, our results highlight a novel role of the CRL regulatory protein CAND1 in centriole duplication control and provide the first evidence of its deregulation in a considerable proportion of primary prostate adenocarcinomas. These findings underscore the frequent disruption of CRL components in human malignancies [13]. Our results also warrant further exploitation for the development of novel predictive biomarkers and innovative therapeutic approaches to target the SCF-CAND1 axis in prostate cancer.

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References

[1] Nigg EA (2006). Origins and consequences of centrosome aberrations in human cancers. *Int J Cancer* **119**, 2717–2723.

[2] Duensing A and Duensing S (2010). Centrosomes, polyploidy and cancer. *Adv Exp Med Biol* **676**, 93–103.

[3] Bornens M (2012). The centrosome in cells and organisms. *Science* **335**, 422–426.

[4] Bettencourt-Dias M, Hildebrandt F, Pellman D, Woods G, and Godinho SA (2011). Centrosomes and cilia in human disease. *Trends Genet* **27**, 307–315.

[5] Lingle WL, Lukasiewicz K, and Salisbury JL (2005). Deregulation of the centrosome cycle and the origin of chromosomal instability in cancer. *Adv Exp Med Biol* **570**, 393–421.

[6] Harrison MK, Adon AM, and Saavedra HI (2011). The G1 phase Cdks regulate the centrosome cycle and mediate oncogene-dependent centrosome amplification. *Cell Div* **6**, 2.

[7] Tsou M-FB, Wang W-J, George KA, Uryu K, Stearns T, and Jallepalli PV (2009). Polo kinase and separase regulate the mitotic licensing of centriole duplication in human cells. *Dev Cell* **17**, 344–354.

[8] Cizmecioglu O, Arnold M, Bahtz R, Settele F, Ehret L, Haselmann-Weiss U, Antony C, and Hoffmann I (2010). Cep152 acts as a scaffold for recruitment of Plk4 and CPAP to the centrosome. *J Cell Biol* **191**, 731–739.

[9] Hatch EM, Kulukian A, Holland AJ, Cleveland DW, and Stearns T (2010). Cep152 interacts with Plk4 and is required for centriole duplication. *J Cell Biol* **191**, 721–729.

[10] Dzhindzhev NS, Yu QD, Weiskopf K, Tzolovsky G, Cunha-Ferreira I, Riparbelli M, Rodrigues-Martins A, Bettencourt-Dias M, Callaini G, and Glover DM (2010). Asterless is a scaffold for the onset of centriole assembly. *Nature* **467**, 714–718.

[11] Cunha-Ferreira I, Rodrigues-Martins A, Bento I, Riparbelli M, Zhang W, Laue E, Callaini G, Glover DM, and Bettencourt-Dias M (2009). The SCF/Slimb ubiquitin ligase limits centrosome amplification through degradation of SAK/PLK4. *Curr Biol* **19**, 43–49.

[12] Korzeniewski N, Zheng L, Cuevas R, Parry J, Chatterjee P, Anderton B, Duensing A, Münger K, and Duensing S (2009). Cullin 1 functions as a centrosomal suppressor of centriole multiplication by regulating Polo-like kinase 4 protein levels. *Cancer Res* **69**, 6668–6675.

[13] Silverman JS, Skaar JR, and Pagano M (2012). SCF ubiquitin ligases in the maintenance of genome stability. *Trends Biochem Sci* **37**, 66–73.

[14] Liu J, Furukawa M, Matsumoto T, and Xiong Y (2002). NEDD8 modification of CUL1 dissociates p120(CAND1), an inhibitor of CUL1-SKP1 binding and SCF ligases. *Mol Cell* **10**, 1511–1518.

[15] Zheng J, Yang X, Harrell JM, Ryzhikov S, Shim EH, Lykke-Andersen K, Wei N, Sun H, Kobayashi R, and Zhang H (2002). CAND1 binds to unneddylated CUL1 and regulates the formation of SCF ubiquitin E3 ligase complex. *Mol Cell* **10**, 1519–1526.

[16] Goldenberg SJ, Cascio TC, Shumway SD, Garbutt KC, Liu J, Xiong Y, and Zheng N (2004). Structure of the CAND1-Cul1-Roc1 complex reveals regulatory mechanisms for the assembly of the multisubunit cullin-dependent ubiquitin ligases. *Cell* **119**, 517–528.

[17] Bosu DR, Feng H, Min K, Kim Y, Wallenfang MR, and Kipreos ET (2010). *C. elegans* CAND-1 regulates cullin neddylation, cell proliferation and morphogenesis in specific tissues. *Dev Biol* **346**, 113–126.

[18] Feng S, Shen Y, Sullivan JA, Rubio V, Xiong Y, Sun T-P, and Deng XW (2004). Arabidopsis CAND1, an unmodified CUL1-interacting protein, is involved in multiple developmental pathways controlled by ubiquitin/proteasome-mediated protein degradation. *Plant Cell* **16**, 1870–1882.

[19] Chew E-H, Poobalasingam T, Hawkey CJ, and Hagen T (2007). Characterization of cullin-based E3 ubiquitin ligases in intact mammalian cells—evidence for cullin dimerization. *Cell Signal* **19**, 1071–1080.

[20] Schmidt MW, McQuary PR, Wee S, Hofmann K, and Wolf DA (2009). F-box-directed CRL complex assembly and regulation by the CSN and CAND1. *Mol Cell* **35**, 586–597.

[21] Lo S-C and Hannink M (2006). CAND1-mediated substrate adaptor recycling is required for efficient repression of Nrf2 by Keap1. *Mol Cell Biol* **26**, 1235–1244.

[22] Piel M, Meyer P, Khodjakov A, Rieder CL, and Bornens M (2000). The respective contributions of the mother and daughter centrioles to centrosome activity and behavior in vertebrate cells. *J Cell Biol* **149**, 317–330.

[23] Korzeniewski N, Cuevas R, Duensing A, and Duensing S (2010). Daughter centriole elongation is controlled by proteolysis. *Mol Biol Cell* **21**, 3942–3951.

[24] Salon C, Brambilla E, Brambilla C, Lantuejoul S, Gazzeri S, and Eymin B (2007). Altered pattern of Cul-1 protein expression and neddylation in human lung tumours: relationships with CAND1 and cyclin E protein levels. *J Pathol* **213**, 303–310.

[25] Mogensen MM, Malik A, Piel M, Bouckson-Castaing V, and Bornens M (2000). Microtubule minus-end anchorage at centrosomal and non-centrosomal sites: the role of ninein. *J Cell Sci* **113**(pt 17), 3013–3023.

[26] Guarguaglini G, Duncan PI, Stierhof YD, Holmström T, Duensing S, and Nigg EA (2005). The forkhead-associated domain protein Cep170 interacts with Polo-like kinase 1 and serves as a marker for mature centrioles. *Mol Biol Cell* **16**, 1095–1107.

[27] Furukawa M, Zhang Y, McCarville J, Ohta T, and Xiong Y (2000). The CUL1 C-terminal sequence and ROC1 are required for efficient nuclear accumulation, NEDD8 modification, and ubiquitin ligase activity of CUL1. *Mol Cell Biol* **20**, 8185–8197.

[28] Duensing A, Liu Y, Perdreau SA, Kleylein-Sohn J, Nigg EA, and Duensing S (2007). Centriole overduplication through the concurrent formation of multiple daughter centrioles at single maternal templates. *Oncogene* **26**, 6280–6288.

[29] Habedanck R, Stierhof Y-D, Wilkinson CJ, and Nigg EA (2005). The Polo kinase Plk4 functions in centriole duplication. *Nat Cell Biol* **7**, 1140–1146.

[30] Murata T, Takayama K, Katayama S, Urano T, Horie-Inoue K, Ikeda K, Takahashi S, Kawazu C, Hasegawa A, Ouchi Y, et al. (2010). miR-148a is an androgen-responsive microRNA that promotes LNCaP prostate cell growth by repressing its target CAND1 expression. *Prostate Cancer Prostatic Dis* **13**, 356–361.

[31] Vogel C and Marcotte EM (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet* **13**, 227–232.